MICROBIOLOGY AND FOOD SAFETY

Prevalence, molecular characterization and antimicrobial resistance of Salmonella serovars isolated from northwestern Spanish broiler flocks (2011–2015)

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ABSTRACT The present study investigated the prevalence, antimicrobial resistance to twenty antibiotics, and class 1 integron and virulence genes of Salmonella isolated from poultry houses of broilers in northwestern Spain between 2011 and 2015. Strains were classified to the serotype level using the Kauffman-White typing scheme and subtyping with enterobacterial repetitive intergenic consensus PCR. The prevalence of Salmonella spp. was 1.02%. Sixteen different serotypes were found, with *S. typhimurium* and *S. arizonae* 48:z4, z23:- being the most prevalent. A total of 59.70% of strains were resistant to at least one, and 19.70% were resistant to multiple drugs. All Salmonella spp. were susceptible to cefotaxime, ciprofloxacin, gentamicin, kanamycin, levofloxacin, neomycin, and trimethoprim. The highest level of resistance was to sulfamethoxazole (40.29%), doxycycline (17.91%), and nalidixic acid (17.91%). None of the isolates carried class 1 integron and only isolates of *S. enterica* subspecies *enterica* were positive for all virulence factors tested, whereas *S. arizonae* lacked genes related to replication and invasion in nonphagocytic cells. This study demonstrates that the prevalence and antimicrobial resistance of Salmonella spp. in poultry houses of broilers of northwestern Spain is low compared with those found in other studies and in other steps of the food chain.

Key words: Salmonella, antimicrobial resistance, class 1 integron, virulence factor, poultry

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INTRODUCTION

Salmonella is one of the most important genera of pathogenic bacteria implicated in food-borne illnesses. In 2013, 85,268 cases of salmonellosis were reported in the European Union (**EU**), 4,537 of which were in Spain, with 76 outbreaks. After *Campylobacter* spp. Salmonella spp. is responsible for most cases of zoonosis, with *S. typhimurium* and *S. enteritidis* being responsible for 74% of human zoonosis cases (EFSA, 2015). However, other subspecies, such as *S. arizonae*, have been related to human infection (Kolker et al., 2012). In addition to eggs and egg products, broiler meat is a major source of human salmonellosis. Therefore, it is necessary to control the presence of Salmonella spp. in poultry farms, and the Commission Regulation (EC) no 200/2012 has set the maximum percentage of broiler flocks that could be positive for *S. enteritidis* and *S. typhimurium* equal to 1% or less (EC, 2012).

Antimicrobial resistance is becoming an important health problem with an alarming increase in the mortality rates from food-borne illness. Multiple drug resistance (**MDR**) has been found in multiple serotypes of Salmonella (Hur et al., 2011; Rothrock et al., 2015). Many antimicrobial resistance genes are present in the Salmonella Genomic Island 1 or in mobile genetic elements as plasmids and integrons that enable the spread of resistance between different strains and the selection of resistant variants in the population (Su et al., 2004). In the *Enterobacteriaceae* family, class 1 integrons are the most prevalent and these integrons can contain different gene cassettes (*pse1, addA1, aadA2*) and are located in Salmonella Genomic Island 1 or are present extrachromosomally (Boyd et al., 2000) and contribute to its transfer.

Salmonella strains can carry virulence factor genes necessary for the invasion of phagocytic and nonphagocytic cells (*invA, sylA*, *sipA*, *spaN*), for survival and replication in the host cells (*pagC*, *sifA*, *spvB*, *spvC*, *ssaR*) and for survival in poor environments (*iroN*). Most of these genes are often located near to each other in the genome, in groups called Salmonella Pathogenicity Islands (Mills et al., 1995). These genes play a specific role in infection by Salmonella in humans and its presence in different strains is a measure of their level of pathogenicity.

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Enterobacterial repetitive intergenic consensus (**ERIC**) PCR, generate species or strain-specific patterns that allow closely related strains to be distinguished. Different studies have shown the utility of ERIC-PCR to efficiently discriminate between Salmonella spp. strains (Campioni et al., 2012; Wang et al., 2014) with a better efficiency than other molecular methods such as Randomly Amplified Polymorphic DNA, Ribotyping PCR, and Single Strand Conformation Polymorphism (Lim et al., 2005).

Therefore, the aim of this study was to evaluate the prevalence and distribution of serotypes of Salmonella in poultry houses, which is the first step of the food chain, of broilers from northwestern Spain between 2011 and 2015. The antimicrobial resistance profiles and presence of resistance and virulence genes were also studied. ERIC-PCR was used to evaluate the genomic polymorphism of Salmonella isolates. To the best of our knowledge, this is the first study related to the prevalence with a systematic sampling frequency and characterization of Salmonella isolates in the northwest of Spain, an important country in the production of poultry meat, with a production of 1,161,000 tons per year (Magrama, 2013).

MATERIALS AND METHODS

Sampling Procedure

A total of 6,577 samples were collected from 371 different poultry houses of broilers in northwestern Spain between 2011 and 2015, with a mean of 110 samples each month and analyzed according to the protocols set by International Organization for Standardization (ISO 6579:2003/A1:2007). Briefly, floor swabs were taken using sterile gauze socks and surfaces were sampled using a sterile cellulose acetate sponge, placed in a sterile plastic bag, and transported to the laboratory immediately. Samples homogenized with 225 mL buffered peptone water (Merck-Millipore, Darmstadt, Germany) and were incubated at 37°C ± 1°C for 18 ± 2 h. Then, 100 μL pre-enriched buffered peptone water was inoculated onto Rappaport-Vassiliadis semi-solid modified medium (Difco Laboratories, Detroit, MI) for selective enrichment at 41.5 ± 1°C for 48 ± 3 h. Plates were grown on xylose lysine deoxycholate agar (Oxoid Ltd., Hampshire, United Kingdom) and on chromID Salmonella agar (SM-ID2, bioMérieux, Marcy-l'Étoile, France) at 37 ± 1°C for 24 ± 3 h.

Confirmation and Serotyping

Putative colonies of Salmonella from xylose lysine deoxycholate and SM-ID2 were purified, cultured in nutrient agar (Merck-Millipore) for 24 ± 3°C at 37 ± 1°C, and confirmed by a latex agglutination test (Microgen, London, UK) and API 20E (bioMérieux). Salmonella spp. were serotyped using the Kauffman-White typing scheme by slide agglutination for the detection of somatic (**O**) and flagelar (**H**) antigens with standard antisera (Biorad laboratories, CA).

ERIC-PCR Typing and Analysis

Purified DNA was extracted with PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, CA) according to the manufacturer's instructions. The ERIC-PCR reactions were carried out in a total volume of 25 μL containing 2.5 μL each primer (5μM), 7.5 μL genomic DNA, and 12.5 μL premix buffer. The primers used were described by Versalovic et al. (1991): ERIC1R (5′-ATGTA AGCTC CTGGG GATTC AC-3′) and ERIC2 (5′-AAGTA AGTGA CTGGG GTGAG CG-3′). The thermocycler program was: initial denaturation at 94°C for 7 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 65°C for 8 min, with a final extension step at 65°C for 10 min. Amplicons were analyzed in a 1.5% agarose gel with SYBR safe (Invitrogen, Leek, The Netherlands) and were visualized with ChemiDoc MP System (Biorad).

A standard weight ladder (TrackIt 100 bp DNA Ladder, InvitrogenTM) was used to normalize the variation between the lanes and gels. Only bands from 150 to 2,000 bp were used. The observed bands were transformed into binary code. A similarity dendrogram was constructed as described Wang et al. (2014) by the UPGMA method using a DICE similarity coefficient with PASW statistics 18 (SPSS Inc, IL). The ERIC-PCR was carried out in triplicate.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was conducted using the broth microdilution method as described in the Clinical and Laboratory Standards Institute (**CLSI**) guidelines (CLSI, 2012). The minimum inhibitory concentrations (**MICs**) for twenty antibiotics were determined: ampicillin (1 to 128 μg/mL), cefotaxime (0.06 to 8 μg/mL), chloramphenicol (2 to 64 μg/mL) cinoxacin (8 to 128 μg/mL), ciprofloxacin (0.06 to 8 μg/mL), doxycycline (1 to 128 μg/mL), enoxacin (0.5 to 32 μg/mL), gentamicin (0.5 to 32 μg/mL), kanamycin (4 to 128 μg/mL), levofloxacin (0.5 to 32 μg/mL), minocycline (1 to 128 μg/mL), nalidixic acid (2 to 64 μg/mL), neomycin (1 to 64 μg/mL), polymixin B (1 to 128 μg/mL), spectinomycin (32 to 256 μg/mL), streptomycin (4 to 256 μg/mL), sulfamethoxazole (8 to 1,024 μg/mL), sulfisoxazole (8 to 1,024 μg/mL), tetracycline (1 to 128 μg/mL), and trimethoprim (0.5 to 32 μg/mL). Standard antimicrobials were obtained from Sigma-Aldrich, except chloramphenicol, gentamicin, kanamycin, and neomycin (Merck-Millipore). *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as control strains and the

Table 1. PCR primers used for amplification of class 1 integron, resistance genes and virulence factors.

Target	Sequence of nucleotides	Reference
Class 1 integron	F-GGCAT CCAAG CAGCA AGC R-AAGCA GACTT GACCT	Mohamed et al. (2014)
invA	F-CTGGC GGTGG GTTTT GTTGT CTTCT CTATT R-AGTTT CTCCC CCTCT TCATG CGTTA CCC	Mezal et al. (2013)
iroN	F-ACTGG CACGG CTCGC TGTCG CTCTAT R-CGCTT TACCG CCGTT CTGCC ACTGC	Mezal et al. (2013)
Sly	F-GCCAA AACTG AAGCT ACAGC TG R-CGGCA GGTCA GCGTG TCGTC G	Guerra et al. (2000)
spiA	F-CCAGG GGTCG TTAGT GTATT GCGTG AGATG R-CGCGT AACAA AGAAC CCGTA GTGAT GGATT	Mezal et al. (2013)
spvB	F-CTATC AGCCC CGCAC GGAGA GCAGT TTTTA R-GGAGG AGGCG GTGGC GGTGG CATCA TA	Mezal et al. (2013)
spvC	F-TGGGG CGGAA ATACC ATC R-GAACT GAGCG CCCAG GCTAA CAC	Mohamed et al. (2014)
pagF	F-TATGA GGATC ACTCT CCGGT A R-ATTCT CCAGC GGATT CATCT A	Mohamed et al. (2014)
Ssa	F-GTTCG GATTC ATTGC TTCGG R-TCTCC AGTGA CTAAC CCTAA CCAA	Campioni et al. (2012)
sifA	F-TTTGC CGAAC GCGCC CCCAC ACG R-GTTGC CTTTT CTTGC GCTTT CCACC CATCT	Mezal et al. (2013)
spaN	F-AAAAG CCGTG GAATC CGTTA GTGAA GT R-CAGCG CTGGG GATTA CCGTT TTG	Mezal et al. (2013)

susceptibility or resistance of each isolate was calculated according to the 2012 CLSI recommendations. The antimicrobials without breakpoint on CLSI guidelines were: neomycin (resistant, $\geq$16 μg/mL; susceptible, $\leq$4), spectinomycin (resistant, $\geq$128 μg/mL; susceptible, $\leq$64), streptomycin (resistant, $\geq$32 μg/mL; susceptible, $\leq$8) according to DANMAP and for polymyxin B (resistant, $\geq$8 μg/mL; susceptible, $\leq$1) according to the SENTRY antimicrobial surveillance program. Isolates that exhibited resistance to at least three classes of antimicrobial agents tested were considered multiresistant.

PCR Detection of Class 1 Integrons, Antibiotic Resistance Genes and Virulence Factors

Purified DNA was extracted as described above and samples were stored refrigerated until use. The PCR reactions were used to investigate the presence of class 1 integrons (*int1*) and virulence genes, using the primers described and listed in Table 1. The PCR conditions were the same as those used in the references. Amplicons were visualized on 2.25% (w/v) agarose gels with SYBR safe DNA gel stain (Invitrogen) using the Chemi-Doc MP System (Biorad).

Statistical Analysis

The antimicrobial resistance levels between the different serotypes isolated were compared using the chi-squared test and Fisher's exact test. Differences were considered significant when probabilities were $P < 0.05$. All statistical analyses were carried out with Statgraphics version XVI.1.15 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Prevalence and Serotypes of Salmonella spp.

The prevalence of Salmonella in poultry houses between 2011 and 2015 was 1.02% (67/6,577). This prevalence is lower than 3.68% recovery in broiler flocks in the EU and 3.29% in Spain according the results reported by EFSA (2015), with the same sampling method that in this study. Also, Salmonella recovery was low compared to other studies in farms and food-chain steps: 14.78% chicken meat from retail stores (Favier et al., 2013), 22.7% of poultry samples from retail stores in Spain (Álvarez-Fernández et al., 2012), 59.3% of layer farms in Korea (Im et al., 2015). This higher recovery may be due to the sampling method or because the poultry environment is less hospitable to survival of this organism. However, multiple factors could contribute to the increased prevalence of Salmonella as food advances through the food chain. Hinton et al. (2004) demonstrated that significant levels of bacterial cross-contamination occur during processing in slaughterhouses and those bacteria that are able to survive processing might multiply in the carcasses during refrigerated storage. Additionally, the EU has established control programs for Salmonella in poultry houses and the results are reflected in a reduction in the prevalence of Salmonella.

Salmonella strains were isolated predominately in autumn (14/67) and winter (44/67) and less in spring (6/67) and summer (3/67). Rodriguez et al. (2006) found a similar annual distribution, with a high prevalence of Salmonella spp. during cold months. Thus, the temperatures of winter and autumn could be better for Salmonella survival. However, data from the EU summary report establish that cases of salmonellosis were

Table 2. Serotypes isolated from poultry houses samples and prevalence in 6,577 samples used.

Salmonella serotype	Isolates	Prevalence %
S. typhimurium	13	0.198
S. arizonae serovar 48:z4,z23:-	11	0.167
S. Anatum	10	0.152
S. arizonae serovar 48:z4,z23,z32:-	8	0.122
S. infantis	6	0.091
S. Newport	5	0.076
S. enterica subspecies *enterica* 4:b:-	3	0.046
S. Bardo	2	0.030
S. Ndolo	2	0.030
S. Canada	1	0.015
S. diarizonae serovar 48:i:z	1	0.015
S. enteritidis	1	0.015
S. salamae serovar 4,12:b:-	1	0.015
S. salamae serovar 4,5,12:b:-	1	0.015
S. salamae serovar 6,8:g,m,t	1	0.015
S. Seftenberg	1	0.015
Total	67	1.017

more frequent in summer, which perhaps is related to bad storage conditions of food or it being poorly cooked.

Strains belonging to four different subspecies were isolated: 64.18% *S. enterica* (43/67), 28.36% *S. arizonae* (19/67), 4.48% *S. salamae* (3/67), and 1.49% *S. diarizonae* (1/67), with the subspecies *enterica* being the most prevalent. The distribution of serovars is shown in Table 2. A total of 16 different serotypes were isolated, and 10 of these belonged to subspecies *enterica*. Four of the isolated serotypes (*S. enteritidis*, *S. infantis*, *S.* Newport, and *S. typhimurium*) are among the top 10 most frequent serovars involved in confirmed cases of human salmonellosis in Europe (EFSA, 2015).

The most prevalent serovars were *S. typhimurium* and *S. arizonae* serovar 48:z4, z23:-, with an incidence of 19.40% (13/67) and 16.41% (11/67), respectively, followed by *S.* Anatum 14.92% (10/67), whereas *S. enteritidis* represented 1.49% (1/67) of strains. *S. typhimurium* and *S. enteritidis* prevalence agrees with the results of EFSA (2015). However, some studies performed in Spain in different places and years (Capita et al., 2007; Álvarez-Fernández et al., 2012) found a high prevalence for *S. enteritidis*. The samples of these studies were obtained from slaughterhouses and retail stores. Thus, *S. enteritidis* might possess some factors that cause it to have a higher prevalence than *S. typhimurium* in the subsequent steps of the food chain. The prevalence of *S. typhimurium* and *S. enteritidis* in analyzed samples was 0.21% (14/6577). This is less than the 1% objective of EU Regulation (CE) nº 200/2012, but is higher than that reported by EFSA in Spain (2015), where the prevalence of *S. typhimurium* and *S. enteritidis* was 0.07%.

S. Anatum was the third serotype isolated in this study. A study carried out by Rodríguez et al., (2006) found a high prevalence of this serotype in poultry farms. It is notable that 28.36% (19/67) of isolates belong to the subspecies *arizonae*, which is typically associated with cold-blooded animals, but other studies (Rodriguez et al., 2006; Evangelopolulou et al., 2014) also found that *S. arizonae* was related to pigs. These studies, and the results of this study show that *S. arizonae* can survive in other environments related to warm-blooded animals. Although *S. arizonae* is an uncommon human pathogen, some infections were reported in immunocompromised patients (Kolker et al., 2012), therefore, it is important not to underestimate the prevalence of this subspecies.

ERIC-PCR

Molecular characterization using ERIC-PCR showed two large clusters (Figure 1); number two containing *S.* Ndolo strains and number one containing the remaining strains. Cluster number one could also be split into two principal groups; group A contained 37 strains, all belonging to *Salmonella enterica* subspecies *enterica* and group B containing 28 strains of the four subspecies present in this study. Four different genetic profiles were found for the 13 *S. typhimurium* strains analyzed. However only two different profiles were obtained for *S. arizonae*, with only one differential band. Most studies involving Salmonella ERIC-PCR were carried out with serotypes from *Salmonella enterica* subspecies *enterica* (Campioni et al., 2012; Wang et al., 2014), where the effectiveness of this method to discriminate between isolates was demonstrated. Nevertheless, pulsed field gel electrophoresis is normally the method of choice for Salmonella typing because has a high discrimination index that ERIC-PCR. However, no studies have been carried out on the subspecies *arizonae* and possibly, the potential to discrimination this isolate is lower than for other serotypes of *Salmonella*.

Antimicrobial Susceptibility Testing

Susceptibility profiles are shown in Table 3. A total of 59.70% (40/67) of Salmonella isolates were resistant to at least one antimicrobial tested, 29.85% (20/67) showed resistance to two or more antibiotics and 19.70% (13/66) of isolates demonstrated MDR. No resistance was observed in 73.68% (14/19) of *S. arizonae* and 53.85% (7/13) of *S. typhimurium* isolates. All strains were susceptible to: cefotaxime, ciprofloxacin, gentamicin, levofloxacin, kanamycin neomycin, and trimethoprim. Resistance to sulfamethoxazole was the most prevalent, at 40.29% (27/67), followed by that to doxycycline (17.91%, 12/67), nalidixic acid (17.91%, 12/67), ampicillin (16.41%, 11/67), minocycline (16.41%, 11/67), streptomycin (16.41%, 11/67), and chloramphenicol (14.92%, 10/67).

Studies performed in Spain showed a high variation in the level of resistance: Álvarez-Fernández et al. (2012) found that all Salmonella isolates were MDR. Carramiñana et al. (2004) found all strains resistant, and Usera et al. (2002) and Capita et al. (2007) found 62.1% and 45% of isolates, respectively, to be resistant to at least one antibiotic. Therefore, cross-resistance

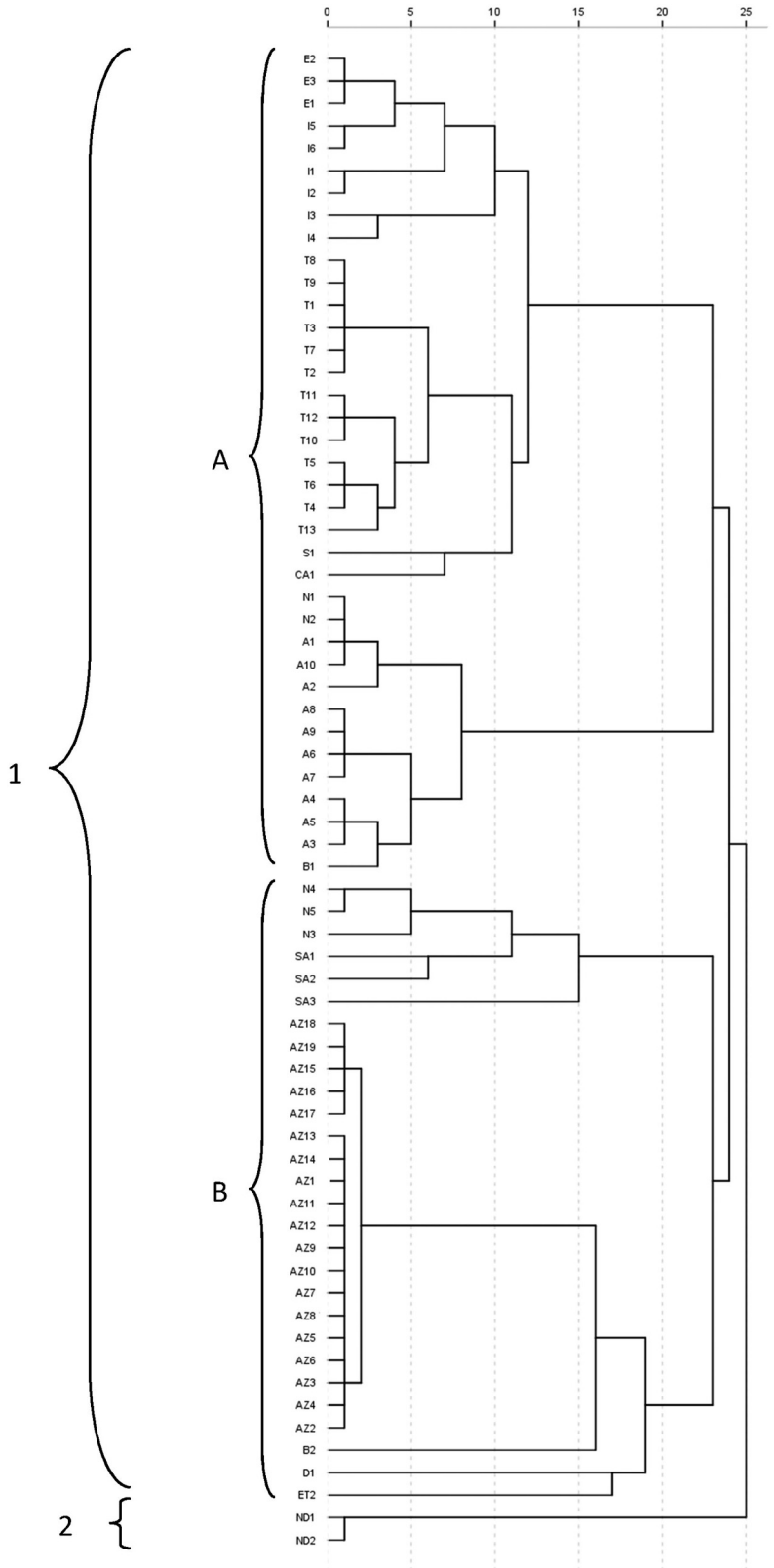
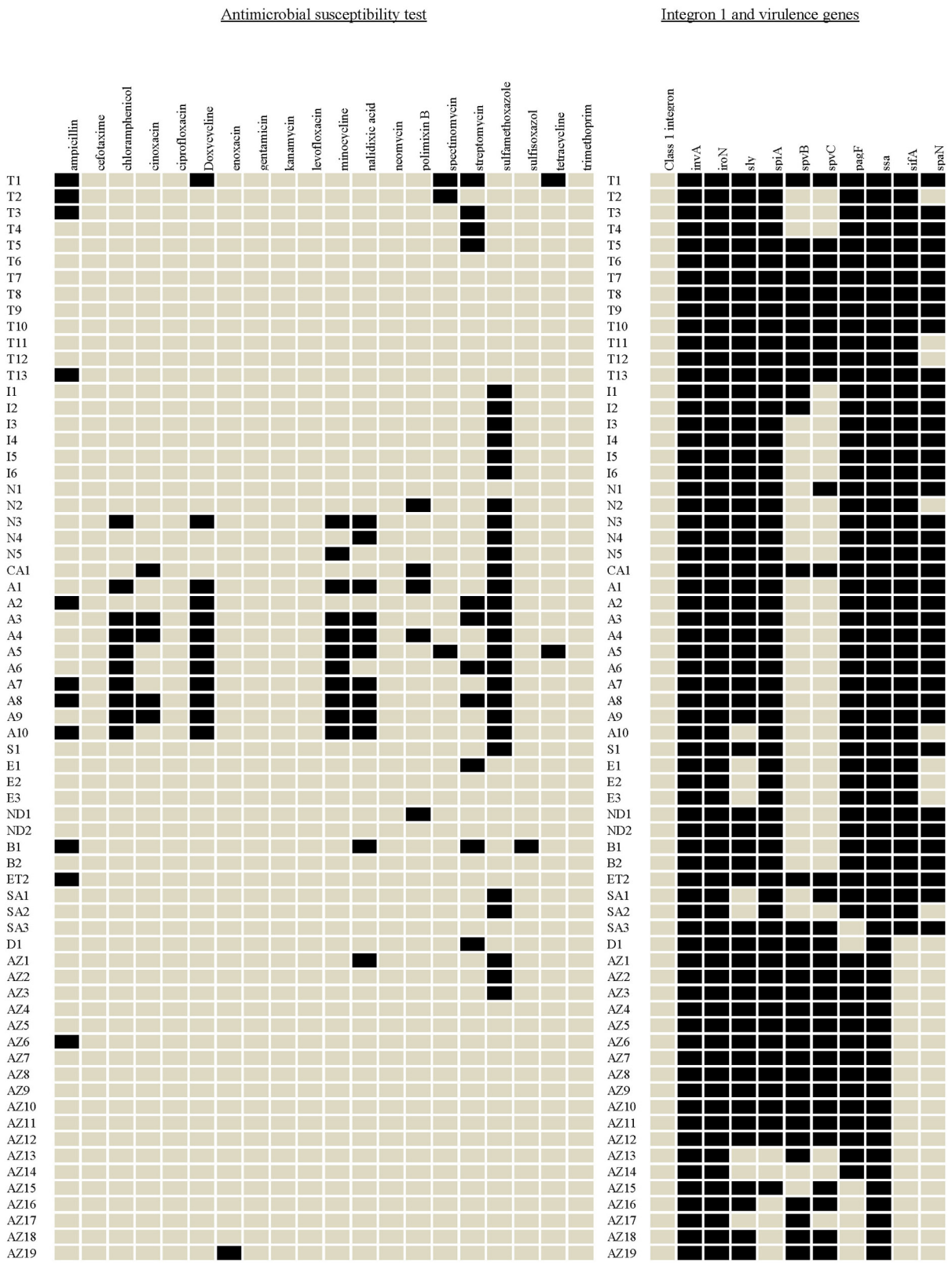


Figure 1. ERIC-PCR Dendrogram showing the relationship between Salmonella isolates. The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method with SPSS program. *S. typhimurium* (T1-T13), *S. infantis* (I1-I6), *S.* Newport (N1-N5), *S.* Canada (CA1), *S.* Anatum (A1-A10), *S.* Seftenberg (S1), *S. enterica* subspecies *enterica* 4:b:- (E1-E3), *S.* Ndolo (ND1-ND2), *S.* Bardo (B1-B2), *S. enteritidis* (ET1), *S.* salamae (SA1-SA3), *S.* diarizonae (D1), *S.* arizonae (AZ1-AZ19).

Table 3. Antimicrobial susceptibility test, black for resistance and grey susceptibility. For virulence and integron black for presence and grey for absence of gene. *S. typhimurium* (T1-T13), *S. infantis* (I1-I6), *S.* Newport (N1-N5), *S.* Canada (CA1), *S.* Anatum (A1-A10), *S.* Seftenberg (S1), *S. enterica* subspecies *enterica* 4:b:- (E1-E3), *S.* Ndolo (ND1,ND2), *S.* Bardo (B1,B2), *S. enteritidis* (ET1), *S salamae* (SA1-SA3), *S. diarizonae* (D1), *S. arizonae* (AZ1-AZ19).



mechanisms might allow the exchange of genetic material between different bacterial genera and confer genes that are required for survival in stress environments, and thus be responsible for the resistances acquired by Salmonella in different environments of the food industry. Similarly, Commission Regulation n° 1831/2003 banned the use of antimicrobials as growth promoters in animal husbandry (EC, 2003). Thus, since 2006, antimicrobial agents are used only for prophylactic or therapeutic purposes. This might lead to reduced resistance, especially if antimicrobials are not used at inhibitory concentrations.

Sulfamides, fluoroquinolones, and tetracyclines are the most common antimicrobials used in chicken flocks in a large number of countries, including Spain (Capita et al., 2007). Thus, a high resistance to sulfamethoxazole was observed and also to tetracyclines, doxycycline, and minocycline. Resistance was found to the quinolones nalidixic acid and enoxacin, with a level of 16.41% and 1.49%, respectively. Resistance to these groups of antimicrobials were found by other authors in Salmonella isolates from poultry (Capita et al., 2007; Voss-Rech et al., 2015). These data suggest that the use of these antimicrobials in broilers can lead to the emergence of resistant strains.

In this study, one of the most common resistances was to streptomycin (16.41%), similar to findings of Usera et al. (2002) and Carramiñana et al. (2004), but resistance to ampicillin and chloramphenicol was also observed. These antibiotics were used over many years, to treat human salmonellosis, but due to the increase in resistance, extended spectrum cephalosporins and fluoroquinolones have been commonly used in humans (Miranda et al., 2009). The resistance to chloramphenicol is notable, because its use has been banned by the EU since the mid-1990s. Sommer & Dantas (2011) reported that the use of antibiotics alters resistance genes encoded by the microbial community and these effects can persist for decades following the last use of the antibiotic. It is difficult to find reports concerning the antimicrobial activity of polymyxin B against Salmonella spp. In previous studies, polymyxin B is an amphipatic polypeptide antimicrobial agent with activity against a wide variety of gram-negative bacilli (Gales et al., 2006). This study found that 7.46% (5/67) of isolates, all belonging to *Salmonella enterica* subspecies *enterica*, were resistant to this antimicrobial.

In this study, isolates belong to subspecies *enterica* carry a mean of 2.35 resistances, whereas isolates from other subspecies possess a mean of 0.43 resistances and significant differences in the number of resistances were observed between both subspecies ($P < 0.05$). In contrast, 65.22% (15/23) of isolates that did not belong to group I were not resistant to at least one antimicrobial tested, whereas 72.77% (32/44) of isolates of the subspecies *enterica* showed resistance, but no significant differences in the number of resistances were found between these two sets of isolates. Evangelopoulou et al. (2014) analyzed 14 *S. arizonae* isolates from pigs and found that they were all resistant to at least to one antibiotic, which differed from the result found in this study. Thus, apart from the subspecies *enterica* from poultry houses, other sub-species can be responsible infrequently for human salmonellosis cases, but these are less problematic with respect to antimicrobial resistance, because 14 isolates showed no resistance and the greatest number of resistances per isolate was two. However, one *S. arizonae* isolate was also resistant to a fluoroquinolone (enoxacin).

All *S.* Anatum isolated were MDR. One isolate of *S.* Bardo, one of *S.* Newport and one of *S. typhimurium* were also MDR. The presence of MDR in all tested *S.* Anatum isolates could be because this strain appears in poultry houses from a human source. Many studies have shown that *S. typhimurium* possesses the highest mean number of antimicrobial resistances (Usera et al., 2002; Capita et al., 2007; Álvarez-Fernández et al., 2012). However, the results of this study showed only one *S. typhimurium* isolate with MDR, two with two resistances and three were only resistant to one antimicrobial, whereas seven isolates were susceptible to all antimicrobials tested. The isolates in this study were sampled from poultry houses, which represent the first step of the food chain, whereas the isolates of the studies cited above were isolated from slaughterhouses and retail stores. Therefore, the different steps of the food chain and food handling by man might be responsible for the spread of *S. typhimurium* with a high level of resistance, coupled with no direct use of antibiotics in poultry houses. The strain *S. enteritidis* was resistant only to ampicillin; other studies have demonstrated that this serotype does not develop resistance as other serotypes (Capita et al., 2007; Álvarez-Fernandez et al., 2012).

Detection of Class 1 Integrons, Resistance and Virulence Genes

Class 1 integrons can encode resistance genes (Cavicchio et al., 2015; Lee et al., 2016). The results showed that none of the isolates tested possessed class 1 integron (Table 3). However, other studies found that gene in Salmonella isolated from poultry sources (Ahmed et al., 2014; Mohamed et al., 2014). The present study showed a low level of antimicrobial resistances in Salmonella compared to those of other studies. The regulated use of antibiotics is possibly responsible for the lower level of resistance genes, because this provides no selective pressure for the maintenance of these genes.

The profile of virulence genes is shown in Table 3. As expected, all strains were positive for the virulence factor *invA*, which is a gene unique to Salmonella, with a highly conserved DNA sequence that has been used to detect Salmonella spp. (Cheng et al., 2009) and is located on a large pathogenicity island on the Salmonella chromosome. The strains were also positive for *iroN*,

which allows Salmonella to acquire iron without a host and also allows Salmonella to be distinguished from other bacteria (Baümler et al., 1997).

In addition, 85.07% (57/67) of strains carried *slyA*. The *slyA* gene encodes an exotoxin that plays an important role in Salmonella survival within macrophages and to oxidative stress (Buchmeier et al., 1997). Therefore, carrying this gene is important for the virulence of Salmonella strains. In total, 89.55% (60/67) and 88.06% (59/67) of isolates were positive for *spiA* and *pagF*, which are genes that allow the survival within macrophages and these results were similar to those found by Mezal et al. (2014) and Mohamed et al. (2014). Only some strains belonging to *Salmonella enterica* subspecies *arizonae* and *Salmonella enterica* subspecies *diarizonae* were negative for these genes. A total of 53.73% of Salmonella were positive for *spaN*, that allows the entry of Salmonella into nonphagocytic cells, and 67.16% of isolates were positive for *sifA*, that allows Salmonella to survive and replicate in host cells (Campioni et al., 2012). All *S. arizonae* and *S. diarizonae* isolates were negative for the *sifA* and *spaN*. These two subspecies are rarely involved in human salmonellosis. The absence of these two genes, which are important for cell invasion and for survival and replication in the host, suggest lower virulence of Salmonella serotypes other than *S. enterica* subspecies *enterica*.

The proportion of isolates positive to *spvC* or *spvB*, were 44.78% and 50.74%, respectively, and 76.92% of *S. typhimurium* isolates were positive for both genes, although three strains were negative for both genes. Among the *S. arizonae* isolates, 84.21% or 89.50% were positive for *spvC* or *spvB*, respectively. These genes are Salmonella plasmid virulence loci, whose expression is important for the replication of Salmonella in the reticuloendothelial system, including the liver and spleen (van Asten and van Dijk, 2005). It is noteworthy that most MDR isolates lack these virulence factors. These genes are usually located on plasmids and are detected if the isolate carries the plasmid. Turki et al. (2012) found all strains of *S.* Kentucky to be negative for this gene. Mezal et al. (2013) and Mezal et al. (2014) found a contrasting pattern for *spvB*; all strains of *S.* Javiana were negative and 96.70% of *S. enteritidis* isolates tested positive. Thus, serotypes known for their virulence, such as *S. typhimurium* and *S. enteritidis* appear to carry this gene more frequently than other serotypes of *Salmonella enterica* subspecies *enterica*. In fact, our results showed that only 10% (3/30) and 6.66% (2/30) of *Salmonella enterica* subspecies *enterica* strains (not *S. typhimurium* or *S. enteritidis*), were positive for *spvB* or *spvC*, respectively. Libby et al. (2002) confirmed the presence of these genes on the chromosome of *S. arizonae*, which explains the high number of positive isolates of *S. arizonae* for this gene.

From the strains tested, 10 out of 67 were positive for all the tested virulence genes; all from *Salmonella enterica* subspecies *enterica*, which is the subspecies more associated with human infections. Within this subspecies, eight strains belonged to *S. typhimurium*, one to *S. enteritidis*, and one to *S.* Canada. Thus, the serotypes related to human disease such as *S. typhimurium* and *S. enteritidis*, showed a high potential virulence.

CONCLUSION

The prevalence of Salmonella in poultry houses from Northwestern Spain was lower than that reported by EFSA, with isolates of *S. enterica* subspecies *enterica* being isolated more frequently than other subspecies. *S. typhimurium* was the serotype found most frequently, and the prevalence of *S. typhimurium* and *S. enteritidis* was less than one percent, which is the target set by the EU. Furthermore, *Salmonella enterica* subspecies *arizonae*, which is normally associated with cold-blooded animals, was isolated at a high frequency from poultry houses. The level of antimicrobial resistance and resistance genes found in the Salmonella spp. isolated from the first step of the food chain was lower than that found in other studies for subsequent steps of the food chain. Therefore, the use of antimicrobials in poultry farms should not be considered the primary factor responsible for the emergence of multiresistant strains, since other factors in the food chain favor the emergence of MDR and the spread of resistance genes. Related to this, none of the strains tested was positive to class 1 integron. The screening of virulence factors showed that only strains belonging to *Salmonella enterica* subspecies *enterica* carry all the tested genes, and other subspecies such as *S. arizone* lack the virulence factors that allow survival and replication in host cells and that are present in other serotypes, thereby decreasing the level of pathogenicity.

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